International Journal of Drug Delivery 7 (2015) 44-51

http://www.arjournals.org/index.php/ijdd/index

Original Research Article

ISSN: 0975-0215

In vitro evaluation of six different mouthwashes against six oral pathogens

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Abstract

Dental caries and throat infections are still considered as serious public health problems and inflict a costly burden to health care services around the world and especially in developing countries. In the present study six mouthwashes were evaluated against six oral microorganisms using turbidity measurements and the antimicrobial effect of each mouthwash was detected by the agar well diffusion method. Of the six mouthwashes tested mouthwash A, B and C emerged as the most effective antimicrobial mouthwashes. Mouthwash C showed the highest effect at the concentrations 50% and 75% by the agar well diffusion method, against four of the oral microorganisms tested. *K. pneumonia* was the mostly affected bacteria showing the highest IZD after treatment with mouthwash C. TEM showed the effect of mouthwash C on *K. pneumoniae* as disruption of bacterial cell membrane and destruction of all internal cell contents. A combination between the most three effective mouthwashes A, B and C was done to investigate their synergestic or antagonistic effects compared to mouthwash C alone. It was surprising that the effect of mouthwash C alone was higher than the other tested combinations.

Keywords: Antimicrobial activity, Chlorhexidine gluconate, Dental caries, Microbial growth inhibition, Mouthwashes, Zone of inhibition.

Introduction

Despite great improvements in the global oral health status in various fields of medicine, dental caries and throat infections still remain the most prevalent diseases around the world [1]. The oral cavity of orthodontic patients undergoes changes characterized by a destruction of superficial dental structures caused by pH reduction which are by product of carbohydrate metabolism by cariogenic bacterium and increased accumulation of food particles. This may lead to an increased number of *Streptococcus mutans* and *Streptococcus sanguinis* colony-forming units in saliva. These bacteria are able to colonize clean and smooth surfaces of teeth. The presence of these bacteria on tooth surfaces increases the possibility of caries development [2].

Due to some of their vital characteristics, *S.mutans* and *S. sanguinis* are regarded potentially highly cariogenic. Therefore, preventive efforts in these risk groups have concentrated on direct suppression of the cariogenic micro flora by chemotherapeutics as an adjunct to improved oral hygiene [3].

Prevention of oral diseases is easier than a cure.The widespread use of mouthwashes as an aid to oral hygiene in the developing countries of the world. Development work on the mouthwashes has been done mostly by the manufacturers, and the little work that has been done relates to the individual ingredients they contain rather than to their complete formulations [4]. Chlorhexidine gluconate (CHX) is the most potent documented antimicrobial agent against *S. mutans* and dental caries [5]. Mouth rinsing with a chemical agent could be a useful clinical adjunct for reducing the bacterial plaque accumulation during the active phase of orthodontic treatment [6].

This study determines and compares the antimicrobial properties of six different types of mouthwashes against six oral pathogens related to caries and throat infections to provide information about the efficacy of these mouthwashes in vitro.

Materials and methods

Mouthwashes

Six brands of mouthwashes were purchased from a pharmacy in Egypt. Their ingredients are shown in Table 1.

Table 1 Active ingredients of various mouthwashes tested for their antimicrobial potential.

Name	Ingredients
A	Chlorhexidine gluconate0.2%, Menthol0.04%,Thymol0.06%, Eucalyptol0.09%, Sodium Fluoride0.02%
B	Tibezonium iodide 50mg
C	ChlorhexidineHcl 125mg
D	DLwater,Propyleneglycol, Glycerin, Potassium Nitrate5%, Levomentholum, Menthaepip, Disodium phosphate, sodium sulfate, sodium saccharin
E	BenzdamineHcl 0.15g
F	Povidone iodine

Microorganisms

Six gram positive and gram negative oral bacteria were used: *Streptococcus mutans* (ATCC497) *Streptococcus sanguinis* (ATCC10556), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogens* (ATCC 19615), *Klebsiella pneumoniae* (ATCC13883) and *Heamophilus influenza* (ATCC 35056). They were subcultured on specific media such as brain heart infusion agar, blood agar and MacConkey's agar media and were incubated at 37 C for 24 hours [7].

Evaluation of mouthwashes

Nutrient broth was prepared containing a 10% concentration of the mouthwashes. They were inoculated with 100µl of the microbial inoculums adjusted at 0.5 McFarland standard and were incubated aerobically at 37 C for 24h. The optical density was measured by spectrophotometer (Humalyzer junior.# 72333, E.E.A) at a wavelength of 640 nm as a guide to microbial growth. The experiments were performed in triplicates. Broth without mouthwash was used as control [8].

Antimicrobial activity

Three different concentrations 1:4(25%), 1:1(50%) and 3:4(75%) were made taking sterile distilled water as the diluents [9]. Muller Hinton agar media was prepared and inoculated with a standardized 0.5 McFarland inoculums of each bacterial strain. A 45µl of each mouthwash concentration was propelled directly into wells made in the inoculated Muller Hinton agar plates. The plates were allowed to stand for ten minutes for diffusion of the mouthwash to take place and incubated at 37 C for 24h [10].

The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the mouthwash, was recorded if the zone of inhibition was greater than 8mm [11]. The mean diameter of inhibition zones was calculated [12].

Transmission electron microscope (TEM) examination

Conventional TEM is frequently selected to visualize the ultra structural damage on both cell wall and cytoplamatic membrane of entire microbes when fixed material can be used [13]. At ultra structural level, a simple negative staining for TEM (JEM-1400 TEM, JEOL- Japan) of bacterial cells can report evidences on the mechanism of membrane disruption by antimicrobial proteins and peptides (AMPPs) [14]. Ultrathin sections obtained by conventional procedures, namely fixation with aldehydes, post-fixation with osmium tetraoxide, dehydratation and embedding in Epoxy resin, allow the observation of membrane and cytoplasmatic alterations. Treatment with AMPPs can induce several external and internal changes such as membrane bleb, ruffling or detachment, the presence of electrodense dots or fibers, hypodense cytoplasmic release and cell vacuolization [15]. The outer membrane detachment observed is generally related to the extremely high affinity of AMPPs to LPS, the main component of the gram-negative bacteria cell wall [15].

Effect of the combination between mouthwashes against the bacterial strains

Combination between the mostly effective mouthwashes at the optimum effective concentrations was done to test the antagonistic/ synergistic inhibitory effect. The test was done by inoculating nutrient broth media containing each tested organism by each combination. All tubes were incubated at 37 C for 24 hours and the optical density was measured by spectrophotometer at wave length 640 nm.

Results

The inhibitory effect of 10% concentration of six different mouthwashes against six oral bacteria was measured by spectrophotometer as optical density (O.D) (Figure. 1). The figure showed that A, B, C were the most effective mouthwashes respectively while D, E, F were the least effective mouthwashes. The inhibition effect of the six mouthwashes were tested at different dilutions 25%, 50% and 75% using agar well diffusion method against *Streptococcus mutans, Streptococcus sanguinis, Staphylococcus aureus, Klebsiella pneumoniae*, *Heamophilus influenza* and *Streptococcus pyogenes* (figure.2).

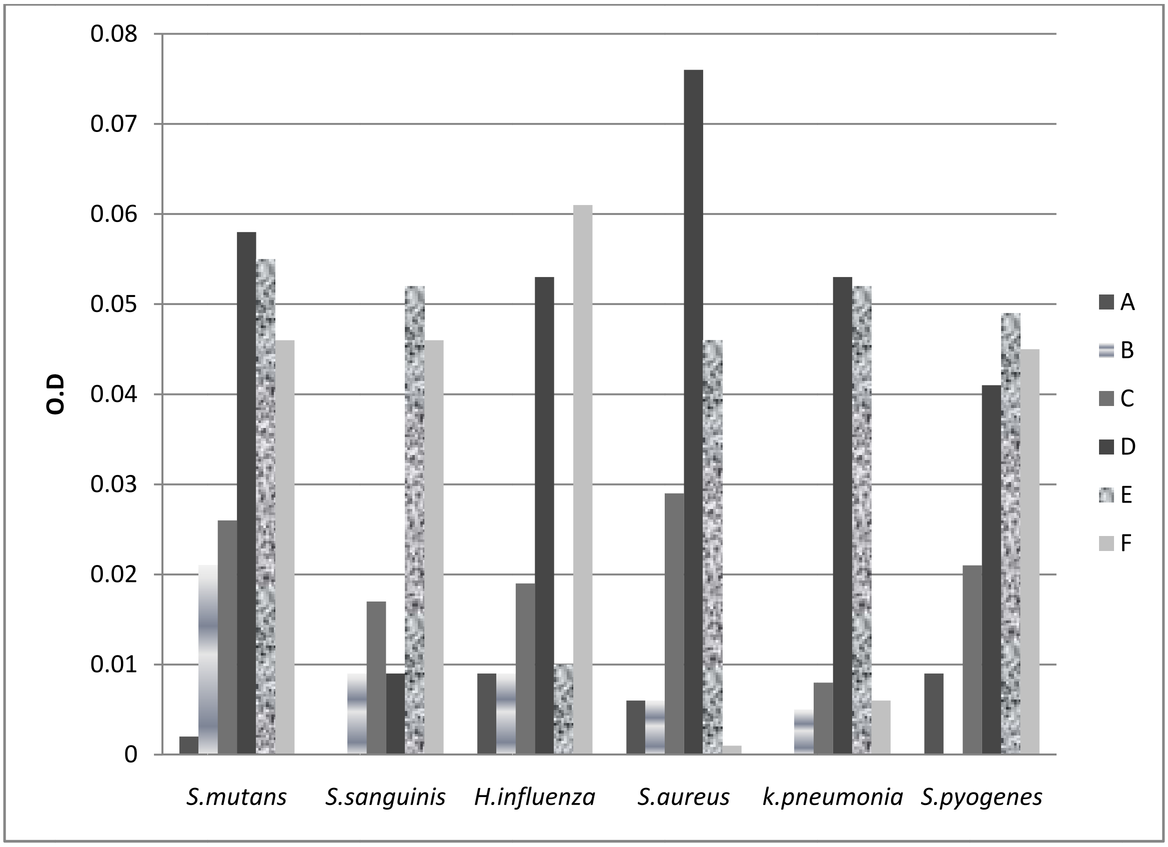
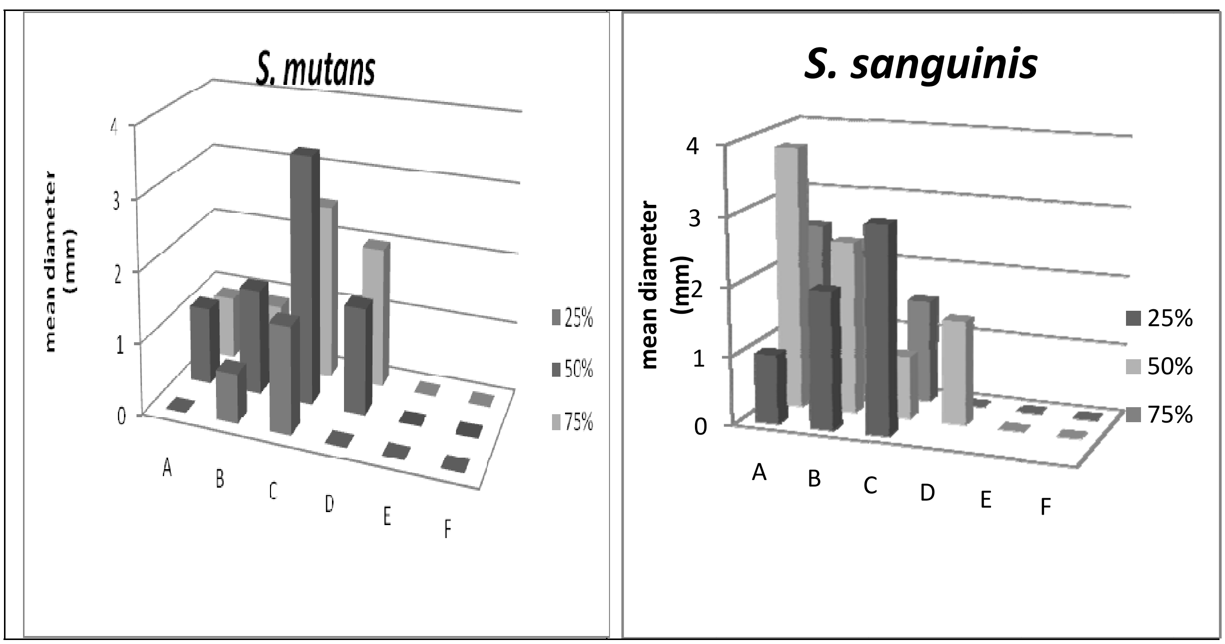


Figure 1 Detection of the antimicrobial effect of the six mouth washes against the six tested microorganisms shown by optical density.



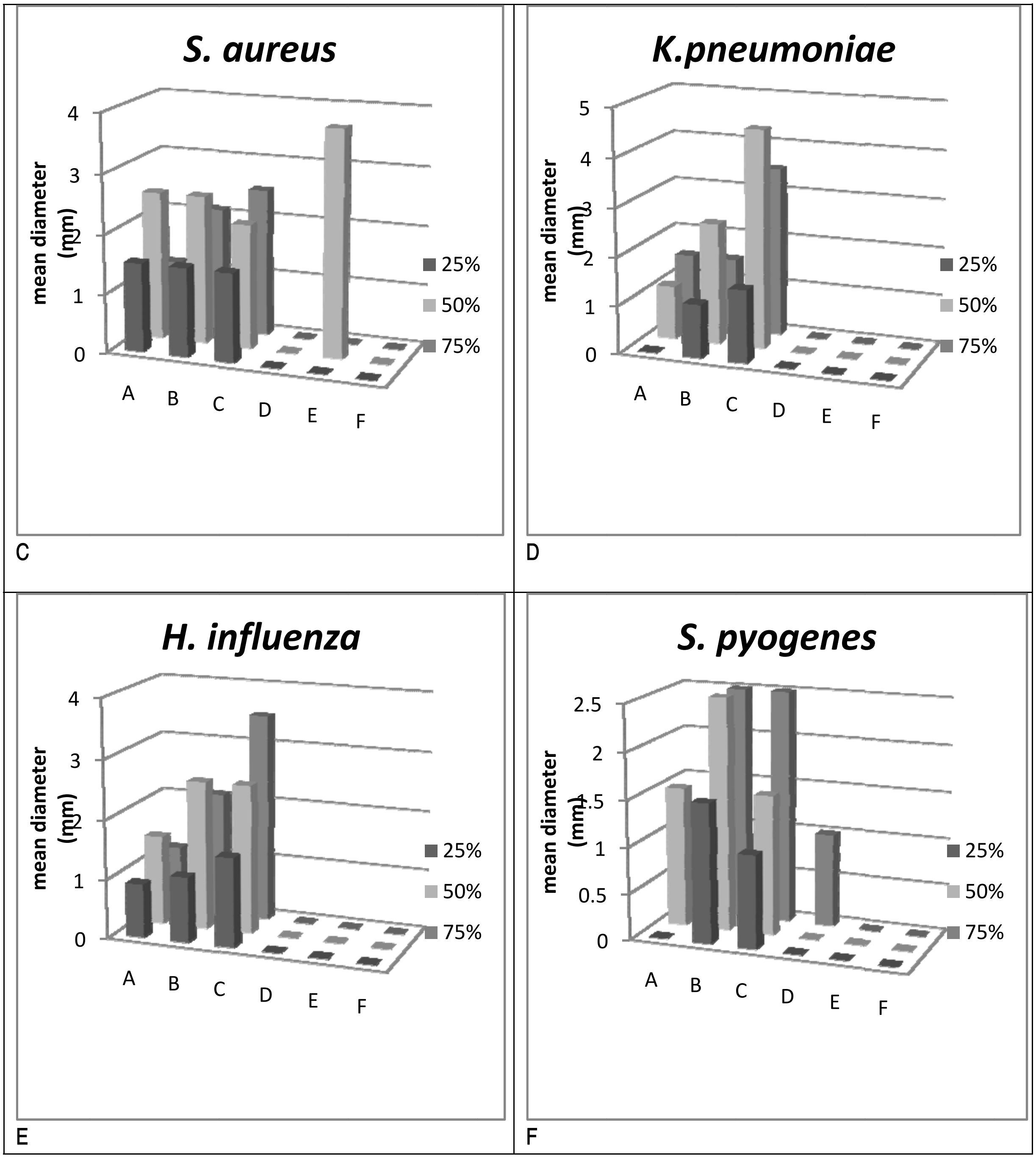


Figure 2 Determination of the inhibitory effect of the six mouthwashes at (25, 50 and 75%) concentrations against *S. mutans* (a), *S. sanguinis* (b), *S. aureus* (c), *K. pneumoniae* (d), *H. influenza* (e) *and S. pyogenes* (f).

It is clear that the most effective mouthwash against *S. mutans* was C at concentration 50% but it was A at concentration 50% against *Streptococcus sanguinis,* while it was E at 50% against *Staphylococcus aureus*. On the other hand the most effective mouth wash against *Klebsiella pneumonia* was C at concentration 50% and also showed the highest effect against *Heamophilus influenza* at concentration 75%. In case of *Streptococcus pyogenes*, the most effective mouthwashs were equally B and C at concentration 75% each.

Figure 2 also indicated that the largest inhibition zone diameter (IZD) was recorded in using mouthwash C at concentration 50% against *Klebsiella pneumonia* (4.5 cm IZD) while mouthwash F showed no effect at all against the six bacterial strains. *Klebsiella pneumoniae* was chosen as the highly affected bacteria using mouthwash C and was scanned by TEM to see the effect of this mouthwash inside the bacterial cell (figure.3).

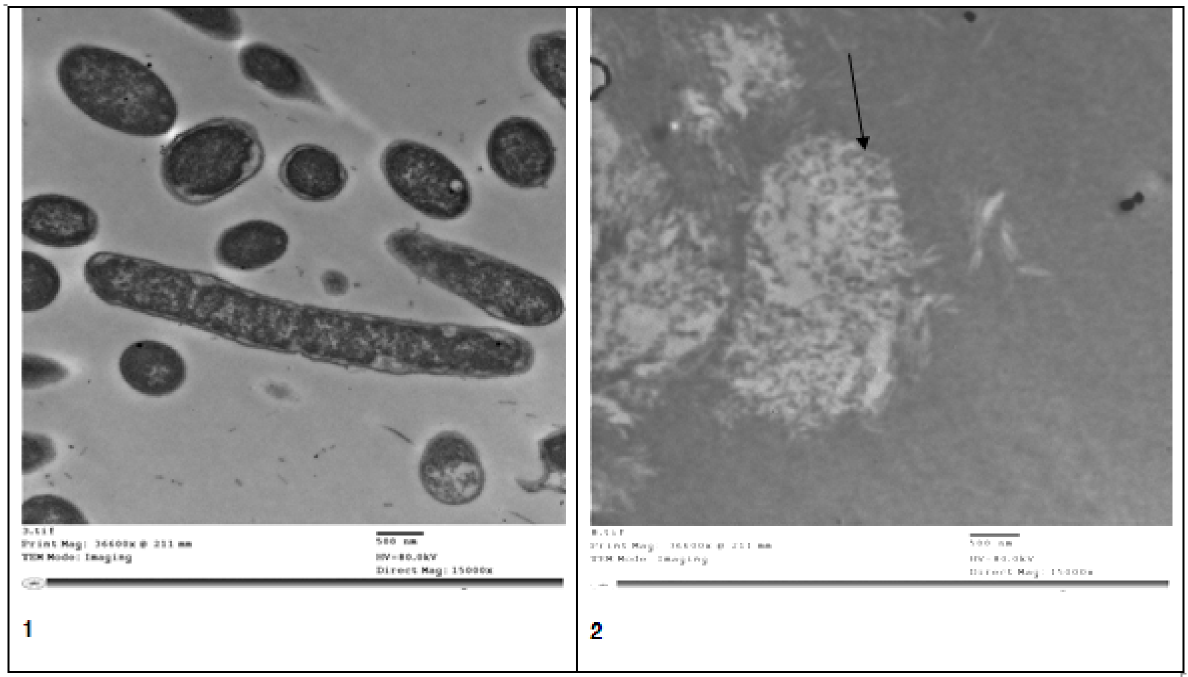


Figure 3 TEM for untreated *Klebsiella pneumonia (*1) and *Klebsiella pneumonia* treated with mouthwash C.

The figure illustrated that treatment of *Klebsiella pneumoniae* with mouthwash C disrupted the bacterial cell and appeared as it is washed out, no membrane and the internal cell content distorted. The cells also appeared larger after treatment which means that the bacterial cells absorbed the mouth wash and destructed.
A combination between the three mostly effective mouthwashes A, B and C were done at the most effective concentration (50%) against the six tested oral bacteria to investigate their synergistic and/or antagonistic effect compared to that of the highly effective mouthwash C alone at concentration 50% by measuring the turbidity (O.D) using spectrophotometer at wavelength 640nm (figure. 4).

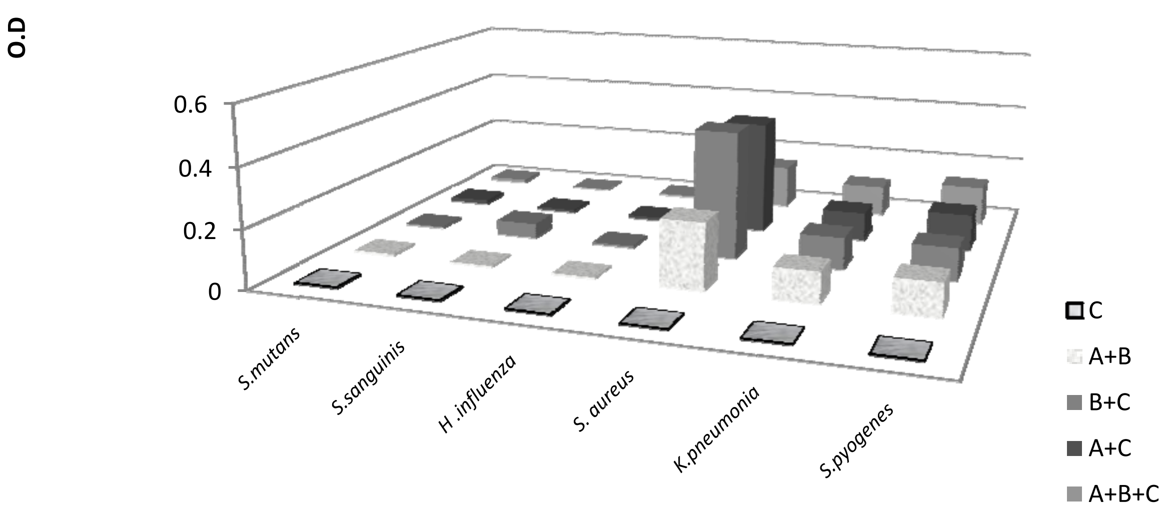


Figure 4 Effect of combination between the highly effective mouthwashes A, B and C compared to the effect of C only.

The figure indicated that mouthwash C showed the highest effect on the six bacterial strains than that of the other combined mouthwashes. Mouthwashes A+C, A+B and A+B+C showed synergistic effect against *Streptococcus mutans, Streptococcus sanguinis* and *Heamophilus influenza* while it showed antagonistic effect against other bacterial strains.

Discussion

It is known that a balance exists in a person's oral microbial population. If this balance is lost, opportunistic microorganisms can proliferate, enabling the initiation of disease processes [16]. In addition, dentists should keep in mind that the mean average inhibition zone of one mouthwash may not be directly comparable with that of another mouthwash because different mouthwashes are constituted of different active ingredients and may diffuse at different rates [17]. In the present study the antimicrobial potential of the six mouthwashes was tested to place the six different mouthwashes in order of antimicrobial effectiveness. The results revealed wide variations in their effectiveness against the six tested microorganisms. Mouthwashes A, B and C emerged as the most effective ones.

More importantly, the test was conducted *in vitro*, so it cannot be assumed that the results of antimicrobial efficacy could be proportional or transferable to the oral cavity and translated into clinical effectiveness. Studies have demonstrated the effectiveness of rinsing with an antimicrobial mouth rinse in significantly reducing both salivary [18-20] and mucosal [21, 22] levels of bacteria.

The findings in this study were in agreement with author [23] who reported that 0.2% chlorhexidine mouthwash decrease *S.mutans* and *S.sanguinis* levels. Also [24] observed that the use of dentifrices containing chlorhexidine seems to be effective for the treatment of gingivitis in orthodontic patients. Other author [25] found a significant reduction of *S. mutans* and *S. sanguinis* in ten patients after using chlorhexidine oral rinse. According to [26], the use of chlorhexidine mouthwash reduces the level of *S. mutans and S. sanguinis*, gingival index and gingival bleeding. The use of chlorhexidine oral rinse contributes to improving oral hygiene in patients with fixed orthodontic appliances [27].

Interestingly, mouthwashes A and C that showed excellent antimicrobial activities contained chlorhexidine as the active ingredient. Chlorhexidine is a cationic biguanide with broad-spectrum antimicrobial action, whose effectiveness in decreasing the formation of dental (plaque) and gingivitis has been demonstrated in several clinical studies [28]. Its mechanism of action is that the cationic molecule binds to the negatively-charged cell walls of the microbes, destabilizing their osmotic balance [29]. This was illustrated in this study by TEM which reveled increasing in bacterial cell volume due to absorption of the mouthwash and no defined internal cell constituent appeared with removal of the bacterial cell membrane. The bacterial cell appeared as fragments collected together with no definite shape.

Thus, from the overall results obtained, it is evident that various mouthwashes listing Chlorhexidine as the active ingredient presented different antimicrobial activities. This is probably due to the different formulations in different mouthwashes in association with other ingredients. The possible explanation may be the active product concentration and its interaction with other constituents, in addition to differences in the formulations, might be responsible for different effects. The result justifies the antimicrobial claims of the mouthwashes, made by earlier workers [8, 30,31].

In this study a combination between the mostly effective mouthwashes was done to show the differences in the effect of mouthwash C alone and its combination with the other two mouthwashes A and B. It was obvious that mouthwash C alone had higher effect on the six oral bacterial strains than combining mouthwash A+B, A+C, B+C and A+B+C. This may be due to antagonistic effect resulted from the differences in the other components added to the active ingredients of each mouthwash which weakened the combination antibacterial effect.

Conclusion

Chlorhexidine formulations are considered to be the "gold standard" antiplaque mouth rinses due to their prolonged broad spectrum antimicrobial activity and plaque inhibitory potential. Mouthwashes A, B and C were the most effective mouthwashes against the six oral bacterial strains tested. Mouthwash A and C contained chlorhexidine as active ingredient. TEM showed that chlorhexidine affected the microbial cell membrane and disrupted the internal cell constituents. The combination between the most effective mouthwashes A, B and C showed antagonistic antibacterial effect compared to that of mouthwash C alone.

Acknowledgment

The author wants to express her deep appreciation to Prof. Abeer A Rushdy, Ain Shams University for her support, guidance and motivation . Deep thanks also for Eiman Abd El- Hameed, October 6th University.

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